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Genetic Investigation of the Catabolic Pathway for Degradation of Abietane Diterpenoids by *Pseudomonas abietaniphila* BKME-9

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We have cloned and sequenced the *dit* gene cluster encoding enzymes of the catabolic pathway for abietane diterpenoid degradation by *Pseudomonas abietaniphila* BKME-9. The *dit* gene cluster is located on a 16.7-kb DNA fragment containing 13 complete open reading frames (ORFs) and 1 partial ORF. The genes *ditA1A2A3* encode the α and β subunits and the ferredoxin of the dioxygenase which hydroxylates 7-oxodehydroabietic acid to 7-oxo-11,12-dihydroxy-8,13-abietadien acid. The dioxygenase mutant strain BKME-941 (*ditA1*::Tn*5*) did not grow on nonaromatic abietanes, and transformed palustric and abietic acids to 7-oxodehydroabietic acid in cell suspension assays. Thus, nonaromatic abietanes are aromatized prior to further degradation. Catechol 2,3-dioxygenase activity of *xylE* transcriptional fusion strains showed induction of *ditA1* and *ditA3* by abietic, dehydroabietic, and 7-oxodehydroabietic acids, which support the growth of strain BKME-9, as well as by isopimaric and 12,14-dichlorodehydroabietic acids, which are diterpenoids that do not support the growth of strain BKME-9. In addition to the aromatic-ring-hydroxylating dioxygenase genes, the *dit* cluster includes *ditC*, encoding an extradiol ring cleavage dioxygenase, and *ditR*, encoding an IclR-type transcriptional regulator. Although *ditR* is not strictly required for the growth of strain BKME-9 on abietanes, a *ditR*::Km^r mutation in a *ditA3*::*xylE* reporter strain demonstrated that it encodes an inducer-dependent transcriptional activator of *ditA3*. An ORF with sequence similarity to genes encoding permeases (*ditE*) is linked with genes involved in abietane degradation.

The pulping of wood to extract fibers used to make paper produces toxic wastewaters (23). The majority of the acute toxicity can be attributed to resin acids found in these wastewaters (18). Resin acids, a class of diterpenoids found in wood extractives, can be grouped into abietanes and pimeranes. Abietane-type acids have an isopropyl chain at the C-13 carbon atom, while pimerane-type acids have methyl and vinyl substituents at this position. Although these compounds are abundant in nature, they are problematic in the pulp and paper industry because of their unnaturally high concentrations in wastewaters. Therefore, resin acids must be removed from wastewater prior to its discharge to the environment. Biological treatment using aerated lagoons is the most common method used for detoxifying the wastewater (19). Several bacteria isolated from such biotreatment systems have been reported to use resin acids as growth substrates, and this catabolic activity appears to be widespread (22, 25).

Pseudomonas abietaniphila BKME-9, a bacterium isolated from a pulp and paper wastewater treatment system, is able to use dehydroabietic acid, an abietane-type resin acid, as a sole source of carbon and reductant (4). A novel three-component aromatic-ring-hydroxylating dioxygenase from this strain has been cloned and expressed (21). In strain BKME-9, dehydroabietic acid is first oxidized by an unidentified enzyme at the C-7 position to yield 7-oxodehydroabietic acid (Fig. 1) (6, 21). The next step in the catabolic pathway involves the DitA aromatic-ring-hydroxylating dioxygenase. This dioxygenase consists of a putative [4Fe–4S] or [3Fe–4S]-type ferredoxin (DitA3) encoded by a gene located 9.2 kb from genes encoding the α and β subunits of the catalytic oxygenase component (DitA1A2) (Fig. 2). DitA activity was reconstituted in *Escherichia coli* and was found to dihydroxylate 7-oxodehydroabietic acid to 7-oxo-11,12-dihydroxy-8,13-abietadien acid. The following enzymatic steps in the dehydroabietic acid degradation pathway have not been characterized in *P. abietaniphila* BKME-9. However, Biellmann et al. (6) purified the 7-oxo-11,12-diol metabolite of dehydroabietic acid from a *Pseudomonas* sp. culture supplemented with the metabolic inhibitor/chelator α,α′-dipyridyl. In a similar study, *Flavobacterium resinovorum* oxidized dehydroabietic acid at C-7 and C-3, followed by decarboxylation at C-4 prior to aromatic-ring hydroxylation (5).

In a previous study, we characterized the DitA dioxygenase encoded by a DNA fragment cloned from strain BKME-9 (21). A transposon (Tn*5*) insertion in the gene encoding the α subunit of the dioxygenase (*ditA1*) resulted in a mutant that had lost the capacity to grow on dehydroabietic acid as well as on the nonaromatic diterpenoid abietic acid. In the course of isolating the gene encoding the ferredoxin component of the dioxygenase (*ditA3*), we cloned and sequenced several open reading frames (ORFs) adjacent to the *ditA1A2* and *ditA3* genes. In this study, we further elucidated abietane degradation by BKME-9 and tested the hypothesis that this strain uses a convergent biodegradation pathway that aromatizes nonaromatic abietanes to dehydroabietic acid or 7-oxodehydroabietic acid prior to aromatic-ring degradation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was cultured on Luria-Bertani medium, and *P. abietaniphila* BKME-9 was cultured on tryptic soy broth, or mineral medium, as previously described (24). Mutants of strain BKME-9 were cultured with 4 μg of gentamicin and/or 30 μg of kanamycin per ml, and *P. abietaniphila* strains harboring derivatives of pUCP26 were cultured with 2 μg of tetracycline per ml.

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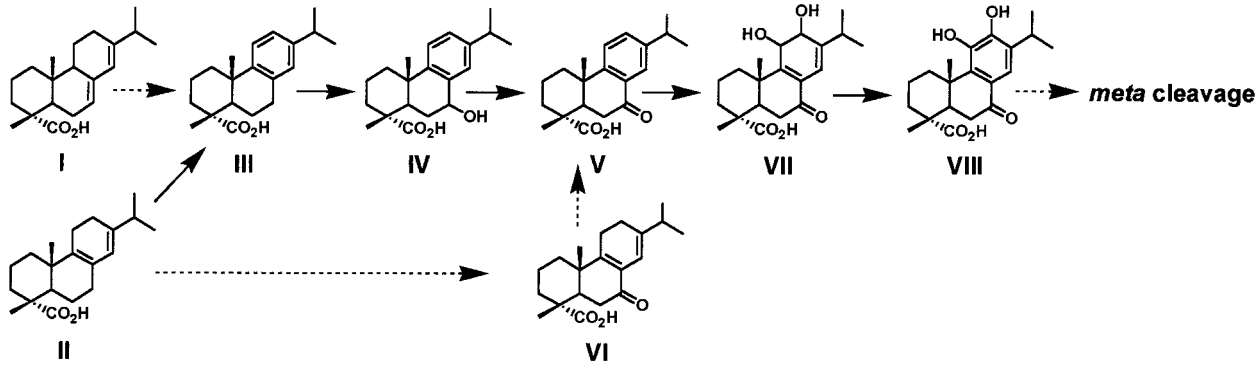

FIG. 1. Proposed pathway for abietanic diterpenoid degradation by *P. abietaniphila* BKME-9. Chemical designations: I, abietic acid; II, palustric acid; III, dehydroabietic acid; IV, 7-hydroxydehydroabietic acid; V, 7-oxodehydroabietic acid; VI, 7-oxopalustric acid; VII, 7-oxo-11,12-dihydroxy-8,13-abietadien acid; VIII, 7-oxo-11,12-dihydroxydehydroabietic acid. Dashed arrows represent hypothetical steps in the pathway.

DNA manipulations and sequence data analysis. The Tn*5* mutagenesis, the construction and screening of the cosmid genomic DNA library from *P. abietaniphila* BKME-9, the subcloning, and the DNA sequencing methodologies were previously described (21). Figure 2 is a schematic representation of the subcloning strategy. Nucleotide sequence analysis was done with Clone Manager for Windows (version 4.01), and PCR primers were designed with Primer Designer (version 2.0). ORFs were analyzed for similarity to GenBank database entries by using the BLASTX and BLASTP programs (1) available on the National Center for Biotechnology Information server via the Internet. Deduced protein sequences were aligned with ClustalX. Searches for PROSITE protein signature consensus sequences and prediction of transmembrane regions were done with the ProScan and TMpred programs available on the Internet server of the Swiss Institute for Experimental Cancer Research.

Insertional inactivation and construction of transcriptional fusions of *dit* genes. The pEX100T gene replacement vector containing the *sacB* counterselectable marker was used for the insertional inactivation of *dit* genes (34). Overhanging ends of DNA fragments required for insertional inactivation were removed with Klenow polymerase or mung bean exonuclease and subcloned into the unique *Sma*I site of pEX100T. The locations of the fragments and the restriction enzymes used to subclone the genes of interest into pEX100T are shown in Fig. 2. To create *xylE* transcriptional insertions/fusions, the *xylE*-Gm^r cassette isolated from pX1918G (without transcriptional terminator) or pX1918GT (with transcriptional terminator) were inserted into the genes in unique restriction endonuclease sites identified in Fig. 2. The cassette containing no terminator was inserted only in those genes suspected of being in a polycistronic transcript (i.e., *ditA3*, *ditB*, *ditD*, and *ditH*), in order to minimize downstream polar effects. The Km^r cassette isolated from a *Sma*I digest of pUC4-KIXX was used to disrupt the putative regulatory gene *ditR*, to allow double mutations in strains carrying the *xylE*-Gm^r fusions. The putative extradiol cleavage dioxygenase gene *ditC*, was inactivated by insertion of the Gm^r gene (without

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
P. abietaniphila		
BKME-9	Wild type	4
BKME-941	*ditA1*::Tn*5*	20
BKME-91	*ditA3*::*xylE*-Gm^r	21
BKME-92	*ditA1*::*xylE*-Gm^r	This study
BKME-93	*ditC*::Gm^r	This study
BKME-94	*ditI*::*xylE*-Gm^r	This study
BKME-95	*ditH*::*xylE*-Gm^r	This study
BKME-96	*ditF*::*xylE*-Gm^r	This study
BKME-97	*ditR*::Km^r	This study
BKME-98	*ditD*::*xylE*-Gm^r	This study
BKME-99	*ditB*::*xylE*-Gm^r	This study
BKME-912	*ditA3*::*xylE*-Gm^r *ditR*::Km^r	This study
E. coli		
DH5α	*endA1 hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*) U169 *deoR* (ϕ80d*lac*Δ (*lacZ*)M15)	Gibco BRL
S17-1	*recA pro thi hsdR* with integrated RP4-2-Tc::Mu-Kan::Tn*7*; Tra$^+$ Tr^r Sm^r	37
Plasmids		
pUC19	Cloning vector, Ap^r	45
pSL1190	Cloning vector, Ap^r	Pharmacia
pEX100T	*sacB* conjugable plasmid for gene replacement; Ap^r	34
pX1918G or GT	*xylE*-Gm^r fusion cassette-containing plasmid; Ap^r Gm^r	34
pUC4-KIXX	Plasmid containing the kanamycin resistance cassette from Tn*5*; Ap^r Km^r	Pharmacia
pUCGm	Plasmid containing the gentamicin resistance cassette; Ap^r Gm^r	33
pUCP26	Conjugable broad-host-range vector; Tc^r	32
pVM220	1,368-bp *Eco*RV-*Bgl*II fragment containing *ditR* cloned into the *Sma*I site of pUCP26	This study
pLC12	SuperCos1 cosmid library clone containing *dit* gene cluster	21
pVM1	5.8-kb *Eco*RI fragment of pLC12 cloned into the *Eco*RI site of pSL1190	21
pVM2	9.8-kb *Eco*RI fragment from pLC12 cloned into the *Eco*RI site of pUC19	21
pVM3	3.2-kb *Sac*II fragment of pLC12 cloned into the *Sma*I site of pUC19	This study
pVM4	3.6-kb *Eco*RI-*Sma*I fragment from pVM2 cloned into the *Eco*RI-*Sma*I site of pUC19	21
pVM5	4.3-kb *Sma*I fragment from pVM2 cloned into the *Sma*I site of pUC19	This study

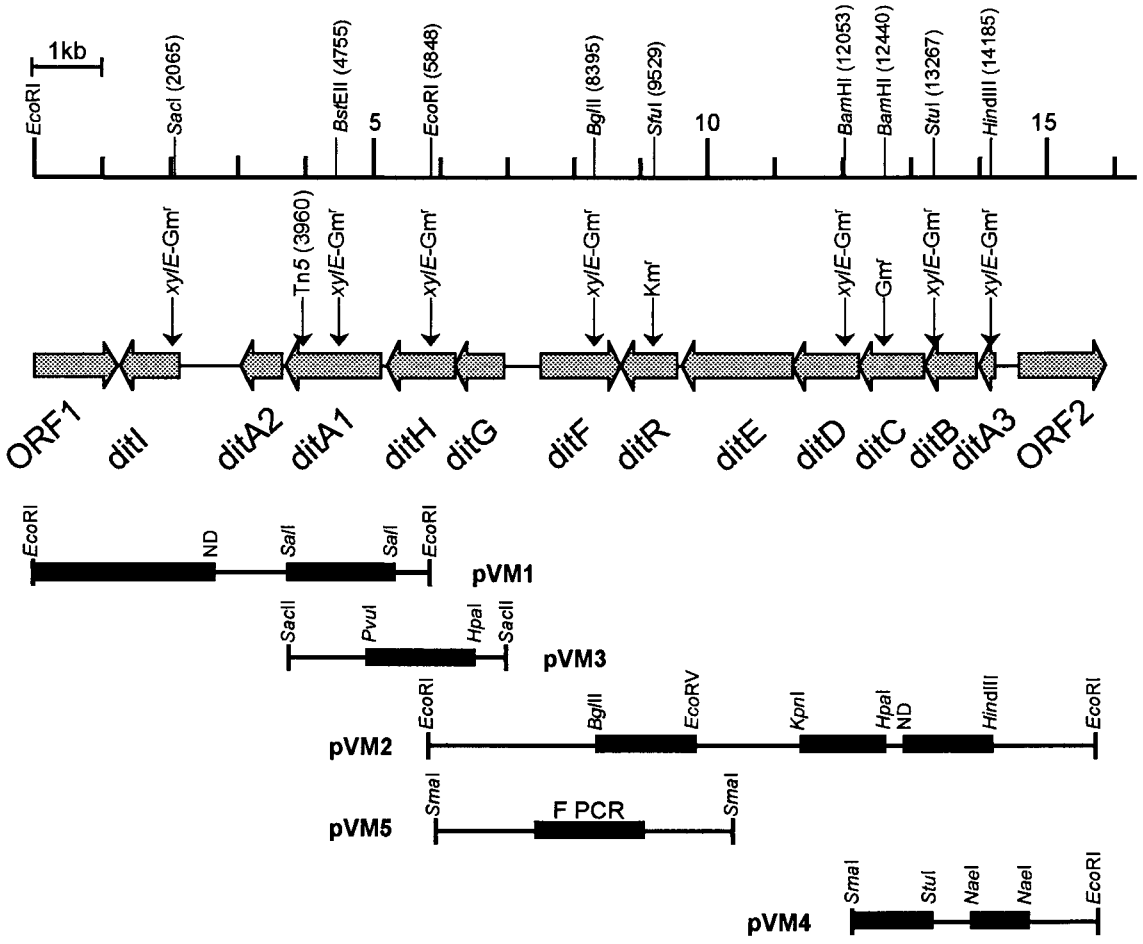

FIG. 2. Physical map of the *dit* gene cluster indicating fragments cloned from the *P. abietaniphila* BKME-9 gene library cosmid pLC12. Solid rectangles, fragments of DNA subcloned for homologous recombination. Arrows indicate the locations of the various insertions, with the endonuclease and base pair positions on the kilobase scale shown. ND, location of nested deletion of clones used in subcloning the DNA fragment. FPCR, fragment of DNA cloned by PCR.

xylE) isolated from a *Bam*HI digest of pUCGm, to avoid possible complementation of the *ditC* mutation by catechol 2,3-dioxygenase (C23O). As several attempts to clone *ditF* using restriction enzymes failed, it was PCR amplified from plasmid pVM5 using primers SCP-1 (5′-TCGAGGATGTCTGGCTG-3′) and SCP-2 (5′-GCTGAGCAAGGTGCTGT-3′) and was cloned into the *Sma*I site of pEX100T. Homologous recombination of the mutated alleles into strain BKME-9 was accomplished by conjugation followed by a two-step selection method, as previously described (21). The gene replacements were confirmed by colony PCR with 17-mer primers at an annealing temperature of 58°C (47).

Phenotypic characterization of *dit* mutant strains. Mutants of strain BKME-9 were characterized for their ability to grow on 0.1 g of either dehydroabietic, abietic, palustric, or 7-oxodehydroabietic acid/liter or on 1 g of pyruvate/liter in a mineral medium. The medium was inoculated (0.1%) with a culture grown overnight on pyruvate and monitored for growth for 3 days. The turbidity caused by the precipitated resin acids in the media made accurate measurement of growth by optical density difficult; therefore, growth was determined by microscopic examinations with comparisons to positive (the wild-type strain, BKME-9) and negative (no substrate) controls. Mutant strains were also tested for the ability to oxidize dehydroabietic acid and accumulate pathway intermediates in cell suspension assays, which were performed as previously described (21). Cell suspensions were monitored by UV-visible light absorption spectroscopy and by gas chromatography as previously described (24), except that samples were acidified with 1 drop of 1 N HCl prior to ethyl acetate extraction in order to improve the recovery of acidic metabolites.

C23O assays. For C23O activity assays, strain BKME-9 was grown on pyruvate to mid-log phase (optical density at 610 nm [OD_{610}], 0.6 to 0.7). Cultures were chilled on ice for 15 min, harvested, and washed twice in 10 mM KPO_4 buffer (pH 7.5) at 4°C. Antibiotics were excluded from the mineral medium, as they reduced the growth rate of the cultures and affected the specific C23O activity. The washed cells were suspended in 10 mM KPO_4 buffer (pH 7.5) and adjusted to a final OD_{610} of 0.6 before induction with 0.5 mM dehydroabietic, abietic, 7-oxodehydroabietic, or isopimaric acid or pyruvate or with 0.1 mM 12,14-dichlorodehydroabietic acid, biphenyl, isopropylbenzene, or phenanthrene. These concentrations were far above the compounds' aqueous solubilities, with the exception of the concentrations of pyruvate and isopropylbenzene. The cell suspensions were incubated with the compounds for 8 h at 30°C on a rotary shaker at 150 rpm, after which C23O activity was measured. All C23O induction experiments were replicated with identical results. Triplicate enzyme assays were performed on whole cells in 1 ml of 10 mM KPO_4 buffer (pH 7.5). C23O activity was assayed spectrophotometrically at 30°C by measuring the formation of 2-hydroxymuconic semialdehyde at 375 nm ($\varepsilon = 44\ mM^{-1}\ cm^{-1}$) for 1 min. Protein concentrations of cell suspensions were determined by using the micro-bicinchoninic acid protein assay kit (Sigma) and bovine serum albumin as the standard (39).

Nucleotide sequence accession number. The nucleotide sequence reported in this study has been submitted to GenBank under accession no. AF119621.

RESULTS

Genetic organization of the *dit* gene cluster and characterization of mutants. The mutagenesis of *P. abietaniphila* BKME-9 with the Tn*5* transposon and the characterization of the diterpenoid aromatic-ring-hydroxylating dioxygenase (21) produced the cosmid clone pLC12, which contains the *dit* gene cluster described in this study. Subcloning of the pLC12 cosmid allowed the sequencing of a 16.7-kb DNA fragment containing 13 complete ORFs and 1 partial ORF (Fig. 2). This fragment was subcloned as two *Eco*RI fragments of 5.8 and 9.8 kb (pVM1 and pVM2), and DNA from pVM2 was further sub-

TABLE 2. Pairwise sequence comparison of deduced amino acid sequences from the *dit* gene cluster with those of similar proteins

Gene	Function or probable function	Deduced no. of residues	Protein with similar sequence	% Identity (no. of residues)	Organism	Accession no.
ORF1	CoA ligase[a]	444 (partial sequence)	FadD	23 (561)	*Escherichia coli*	AE000275
			CaiC	23 (522)	*Escherichia coli*	P31552
ditI	Dehydrogenase/reductase[a]	285	FabG	39 (246)	*Bacillus subtilis*	U59433
			TsaC	35 (252)	*Comamonas testosteroni*	U32622
			BudC	34 (256)	*Klebsiella pneumoniae*	Q48436
			CmtB	32 (259)	*Pseudomonas putida* F1	U24215
ditA2	β Subunit of the ring-hydroxylating dioxygenase	201	BphE	41 (186)	*Comamonas testosteroni* B-356	U47637
			BphE	38 (188)	*Burkholderia cepacia* LB400	P37334
			BphA2	34 (187)	*Rhodococcus* sp. strain RHA1	D32142
			IpbA2	33 (187)	*Rhodococcus erythropolis* B00D2	U24277
ditA1	α Subunit of the ring-hydroxylating dioxygenase	469	BpdC1	31 (461)	*Rhodococcus* sp. strain M5	U27591
			BphA1	30 (460)	*Rhodococcus* sp. strain RHA1	D32142
			TecA1	28 (449)	*Burkholderia* sp. strain PS12	U78099
			TcbAa	28 (450)	*Pseudomonas* sp. strain P51	U15298
ditH	Isomerase/decarboxylase[a]	336	HpaG	20 (429)	*Escherichia coli*	Z37980
			HpcE	19 (405)	*Escherichia coli*	P37352
ditG	Dehydrogenase/reductase[a]	244	PhbB	35 (248)	*Pseudomonas* sp. strain 61-3	AB014757
			FabG	29 (244)	*Bacillus subtilis*	U59433
			2BHD	29 (255)	*Streptomyces exfoliatus*	640224
ditF	Sterol carrier-like protein[a]	397	AcaB	27 (388)	*Pyrococcus furiosus*	X85250
			SCP-X	18 (547)	*Homo sapiens*	U11313
ditR	IclR-type transcription regulator	273	KdgR	22 (263)	*Escherichia coli* K-12	P76268
			GylR	22 (254)	*Streptomyces griseus*	P22866
			PcaR	19 (291)	*Pseudomonas putida* PRS1P	L33795
ditE	Permease of the major facilitator superfamily[a]	547	TetV	23 (419)	*Mycobacterium smegmatis*	AF030344
ditD	Isomerase/decarboxylase[a]	293	HpaG	21 (429)	*Escherichia coli*	Z37980
			HpcE	19 (405)	*Escherichia coli*	P37352
ditC	Extradiol cleavage dioxygenase	313	CarB2	33 (297)	*Pseudomonas* sp. strain CA10	D89065
			BphC	32 (298)	*Burkholderia cepacia* LB400	X66122
			ED02	30 (305)	*Sphingomonas* sp. strain RW1	AJ223219
			EtbC	28 (303)	*Rhodococcus* sp. strain RHA1	D76438 0
ditB	Dehydrogenase/reductase[a]	253	DHG	33 (260)	*Bacillus subtilis*	P12310
			7HSD	28 (267)	*Clostridium sordellii*	P50200
ditA3	Ferredoxin component of ring-hydroxylating dioxygenase	78	T1 Fd	40 (60)	*Thermococcus litoralis*	P29604
			SoyB	34 (65)	*Streptomyces griseus*	P26910
			PhdC	33 (69)	*Nocardioides* sp. strain KP7	AB017795
ORF2	Permease of the major facilitator superfamily[a]	426	GudT	18 (455)	*Escherichia coli* K-12	AE000362

[a] Putative gene products deduced from similarity to other genes with known function.

cloned as 3.6-kb *Eco*RI-*Sma*I (pVM4) and 4.3-kb *Sma*I-*Sma*I (pVM5) fragments (Fig. 2). The complete sequence of ORF2 was obtained by sequencing directly from cosmid pLC12 with custom primers designed from known sequence.

The *dit* DNA cluster of strain BKME-9 comprises genes that encode catabolic and regulatory elements of the diterpenoid degradation pathway, as well as a putative permease (Table 2). We have previously demonstrated that this genomic region encodes the oxygenase (*ditA1A2*) and the ferredoxin (*ditA3*) components of a diterpenoid aromatic-ring-hydroxylating dioxygenase (21). In order to establish the function of other ORFs found in this gene cluster, insertional mutations of nine genes were constructed by homologous recombination. The mutants were characterized for their abilities to accumulate pathway intermediates from dehydroabietic acid and for their abilities to grow on dehydroabietic, palustric, abietic, and 7-oxodehydroabietic acids, four abietane diterpenoids which support the growth of the wild-type strain, BKME-9 (4). Since ^{14}C-labeled abietanes are not commercially available and are difficult to synthesize, we could not perform substrate uptake assays. It was therefore decided not to investigate the function of the two putative permeases, *ditE* and ORF2.

Insertional mutations in 6 ORFs prevented growth on dehydroabietic acid, whereas mutations in 3 ORFs did not prevent growth on this substrate and did not markedly change the growth rate (Table 3). It should be noted that the location of the *xylE*-Gm^r cassette insertion in *ditB* was close to the C terminus (17 residues away) and may not have prevented the expression of a functional enzyme. Interestingly, all mutants which lost the capacity to grow on dehydroabietic acid also

TABLE 3. Phenotypic characterization of *P. abietaniphila* mutants

Strain	Gene mutated	Growth on the indicated substrate[a]				
		Dehydroabietic acid	Abietic acid	Palustric acid	7-Oxodehydroabietic acid	Pyruvate
BKME-9	None	+	+	+	+	+
BKME-94	*ditI*	−	−	−	−	+
BKME-941	*ditA1*	−[b]	−[b]	−	−[b]	+[b]
BKME-95	*ditH*	−	−	−	−	+
BKME-96	*ditF*	−	−	−	−	+
BKME-97	*ditR*	+	+	+	+	+
BKME-98	*ditD*	+	+	+	+	+
BKME-93	*ditC*	−	−	−	−	+
BKME-99	*ditB*	+	+	+	+	+
BKME-91	*ditA3*	−[b]	−[b]	−	−[b]	+[b]

[a] +, growth; −, no growth.
[b] Data previously published in reference 21.

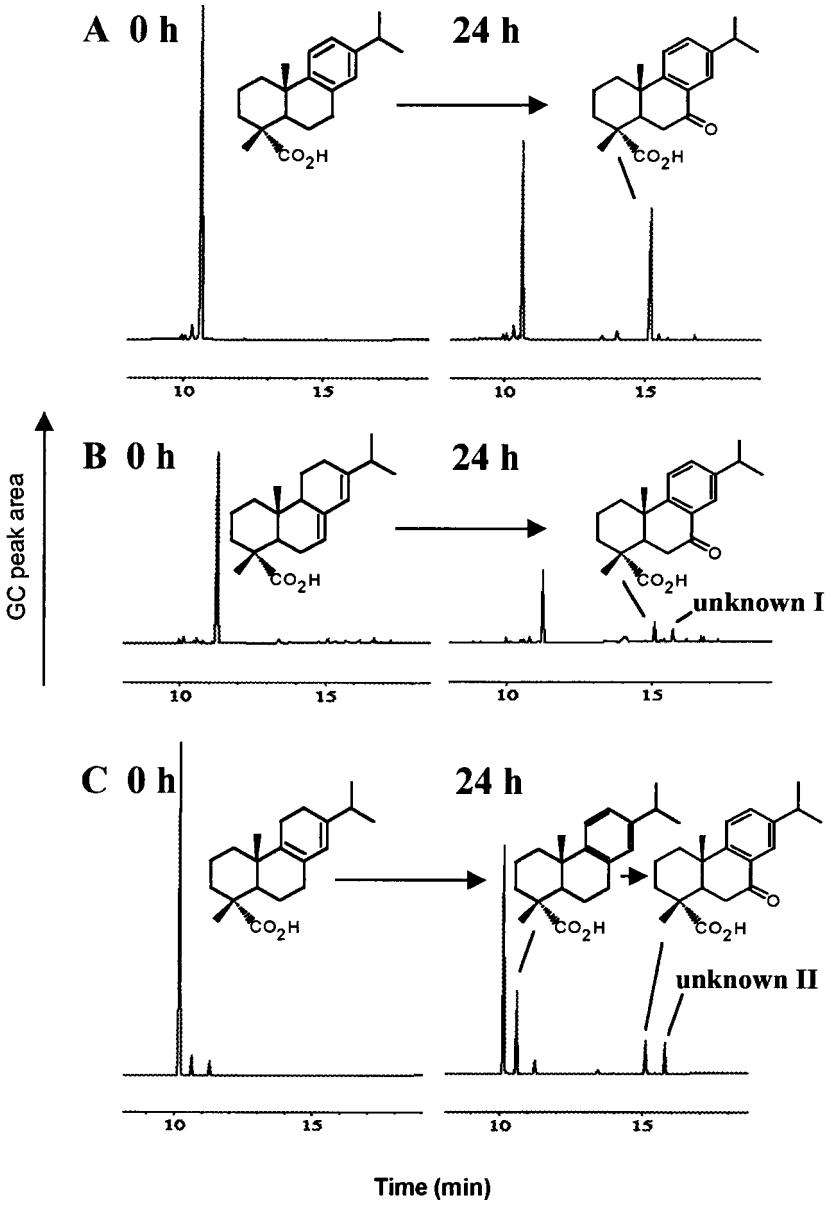

FIG. 3. GC-FID analysis of the dehydroabietic (A), abietic (B), and palustric (C) acid biotransformation products of *P. abietaniphila* BKME-941 (*ditA1*::Tn*5*) at 0 and 24 h of incubation.

failed to grow on the nonaromatic diterpenoids palustric and abietic acids as well as on the pathway intermediate 7-oxodehydroabietic acid (Table 3). These results indicated that a common pathway may be used for the biodegradation of these four diterpenoids. Furthermore, the loss of growth of the mutants on all four substrates also suggested that none of the mutated genes encode enzymes required for the transformation (aromatization) of the nonaromatic abietic and palustric acids to dehydroabietic acid or for the oxidation of dehydroabietic acid to the pathway intermediate 7-oxodehydroabietic acid (Fig. 1).

Evidence of a convergent pathway for abietane degradation. Strains with mutations in two of the genes coding for the aromatic-ring-hydroxylating dioxygenase (*ditA1* or *ditA3*) accumulated the pathway intermediate 7-oxodehydroabietic acid in cell suspension assays with dehydroabietic acid as the substrate (Fig. 3A) (21). Since the disruption of genes encoding this enzyme also resulted in the loss of growth on the nonaromatic substrate, abietic acid (21), we hypothesized that strain BKME-9 aromatizes abietic acid to dehydroabietic acid prior to aromatic-ring attack. To test this hypothesis, the *ditA1*::Tn*5* mutant strain BKME-941 was incubated in cell suspension assays in the presence of abietic and palustric acids, two nonaromatic abietane diterpenoids. Gas chromatography (GC) analyses showed that 7-oxodehydroabietic acid was produced from both substrates and that dehydroabietic acid was produced from palustric acid (Fig. 3B and C). Although it is likely that dehydroabietic acid is an intermediate in the conversion of abietic acid to 7-oxodehydroabietic acid, dehydroabietic acid was not observed when abietic acid was used as the substrate for the mutant strain BKME-941 (Fig. 3B). Two unknown compounds, one from abietic acid and the other from palustric

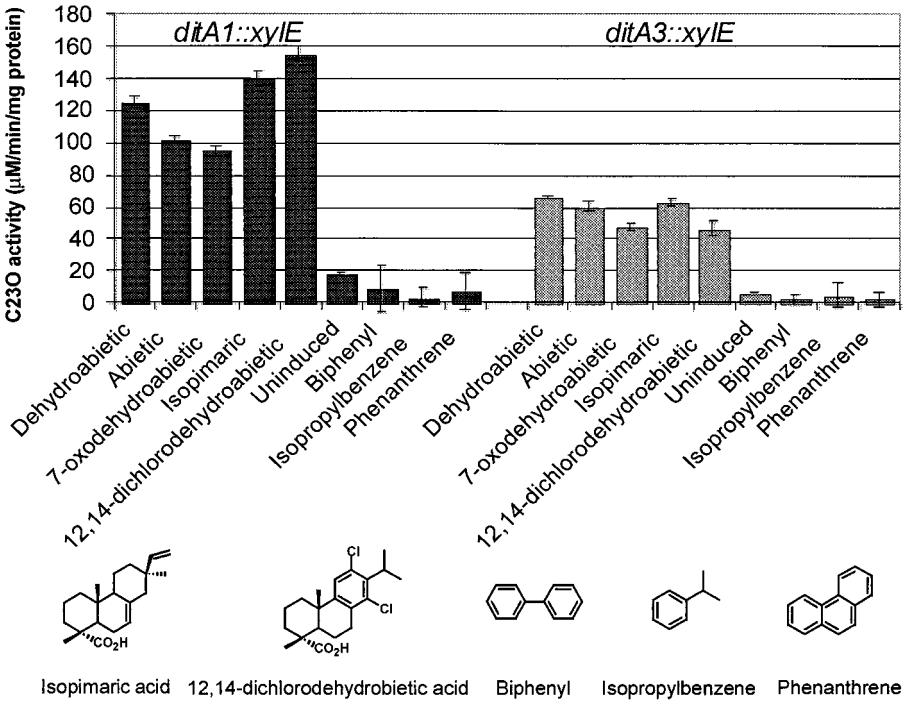

FIG. 4. Expression of *ditA1* and *ditA3* in response to various diterpenoids and aromatic compounds. Compounds were added at 0.5 mM (dehydroabietic acid, abietic acid, 7-oxodehydroabietic acid, and isopimaric acid) and at 0.1 mM (dichlorodehydroabietic acid, biphenyl, isopropylbenzene and phenanthrene). Activity values are means of three enzyme assays ± standard deviations.

acid, were also observed. Although it was not identified with certainty, we suspect that unknown II (Fig. 3C) is the 7-oxo derivative of palustric acid. GC-mass spectrometry (MS) analysis of the culture supernatant showed that the mass spectrum of the methyl ester derivative of unknown II had a fragmentation pattern similar to that of 7-oxodehydroabietic acid with an additional mass of *m*/*z* 2 for the molecular ion at *m*/*z* 330 (19% relative intensity) and a base peak of *m*/*z* 255 (7-oxodehydroabietic acid has a molecular ion of *m*/*z* 328 and a base peak of *m*/*z* 253). This compound was therefore tentatively identified as 7-oxopalustric acid (Fig. 1, compound VI). The identity of unknown I could not be determined from its mass spectrum alone. Approximately 90% of the carbon was recovered as substrate or identifiable metabolites after 24 h of incubation with dehydroabietic or palustric acid. The carbon mass balance was poor for abietic acid, with approximately 40% recovery. However, abiotic controls also showed a loss of 22% of the abietic acid carbon, indicating that this compound was somewhat unstable under the assay conditions. The transformation of abietic and palustric acids to dehydroabietic acid or isomerization of palustric to abietic acid did not occur in the abiotic incubations of the substrates in mineral medium, despite the fact that these reactions have been previously reported to occur spontaneously under some conditions (40). The inability of the strain BKME-9 mutants to grow on nonaromatic compounds (Table 3) and the metabolites identified in cell suspension experiments (Fig. 3) demonstrated that the aromatization of abietic and palustric acids to dehydroabietic and/or 7-oxodehydroabietic acid is essential to this biodegradation pathway for abietane-type diterpenoids.

Analysis of *ditA1* and *ditA3* gene expression and inducer specificity. The expression of the genes encoding the α subunit and ferredoxin components of the diterpenoid dioxygenase was analyzed by measuring C23O activities of *ditA1*::*xylE* (strain BKME-92) and *ditA3*::*xylE* (strain BKME-91) transcriptional fusion strains. Cultures of the wild-type strain, BKME-9, grown on pyruvate and subsequently induced with each of five diterpenoids showed no endogenous C23O activity, which demonstrated that *xylE* was an adequate reporter gene for induction studies of this pathway. Uninduced cultures of the reporter strains BKME-91 and BKME-92 showed basal levels of C23O expression (Fig. 4), indicating that the expression of *ditA1* and *ditA3* was leaky. The specificity of induction of these genes was investigated by using dehydroabietic, abietic, and 7-oxodehydroabietic acids, which are growth substrates for strain BKME-9, as well as 12,14-dichlorodehydroabietic and isopimaric acids, which are not metabolized by BKME-9 (25). Surprisingly, all five diterpenoids induced *ditA1* and *ditA3* expression (Fig. 4). Since we have demonstrated that abietic and dehydroabietic acids are transformed to 7-oxodehydroabietic acid by *ditA1* and *ditA3* mutants (Fig. 3), it is plausible that only 7-oxodehydroabietic acid, the substrate for the dioxygenase, is the inducer and not abietic or dehydroabietic acids. However, considering the high C23O levels observed with the nonmetabolizable diterpenoids 12,14-dichlorodehydroabietic and isopimaric acids, we believe that *ditA1* and *ditA3* expression was directly inducible by abietic and dehydroabietic acids. Induction of the aromatic-ring-hydroxylating dioxygenase appears to require diterpenoids, as the aromatic compounds biphenyl, isopropylbenzene, and phenanthrene, which are not growth substrates for strain BKME-9 (25), did not induce *ditA1* or *ditA3* expression (Fig. 4).

Regulation of *ditA3* expression by DitR. Located between *ditA3* and *ditA1*, 4.4 kb downstream of the former gene and 3.8 kb upstream of the latter gene, an ORF was identified with similarity to regulatory proteins (Table 2). This ORF, designated *ditR*, is located 79 nucleotides downstream of *ditE*. Comparison of the deduced amino acid sequence of DitR to the

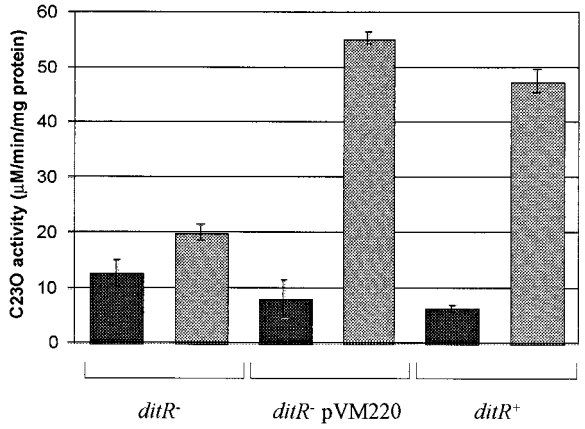

FIG. 5. Expression of C23O by the *ditA3*::*xylE* reporter strain BKME-91 (DitR$^+$), by the *ditA3*::*xylE ditR*::Km double mutant strain BKME-912 (DitR$^-$), and by strain BKME-912 harboring a plasmid containing *ditR* (DitR$^-$ pVM220). Cultures were either not induced (solid bars) or induced with 0.5 mM 7-oxodehydroabietic acid (shaded bars). Activity values are means of three enzyme assays ± standard deviations.

GenBank database entries revealed low sequence identity to IclR-type transcription regulators (41) (Table 2). In addition to the sequence similarity, we also identified a potential helix-turn-helix (HTH) DNA binding motif by using the method of Dodd and Egan (10), further suggesting that *ditR* encodes a regulatory protein. Like regulators of the IclR-family, the HTH motif (53-SVDLARVLGINPSTCFNILR-71) of DitR is located in the N-terminal region of the deduced protein. To determine if DitR regulates the expression of the ferredoxin gene, the *ditR* gene was inactivated in the C23O reporter strain, BKME-91. A Km^r cassette was inserted in *ditR* of BKME-91, and the selection of Gm^r Km^r double mutants yielded strain BKME-912 (*ditA3*::*xylE*-Gm^r *ditR*::Km^r). C23O assays of strain BKME-912 induced with 7-oxodehydroabietic acid showed that the expression level of *ditA3* was similar to noninduced levels (Fig. 5). Electroporation of pVM220, a *ditR*-containing plasmid, into strain BKME-912 restored the transcriptional regulation of *ditA3*, as shown by its wild-type expression level (Fig. 5). Thus, DitR positively regulates expression of *ditA3*, and a *ditR* knockout mutant can be complemented in *trans*.

The extradiol ring cleavage dioxygenase DitC. The *ditC* gene encodes an extradiol ring cleavage dioxygenase. Cloning of a 1.2-kb fragment containing *ditC* into pEX100T, under the control of the *lac* promoter in the heterologous host *E. coli*, resulted in clones capable of the extradiol cleavage of 2,3-dihydroxybiphenyl (2,3-DHB), as indicated by the formation of yellow colonies in spray plate assays. However, this ring cleavage dioxygenase activity was not observed when the cosmid library clone pLC12 containing *ditC* was tested with 2,3-DHB. The latter result might be explained by a lack of *ditC* expression from its native promoter in *E. coli* or by an undetectable activity due to low cosmid copy number combined with low activity towards 2,3-DHB. Amino acid sequence analysis of DitC revealed that it is a two-domain type I extradiol dioxygenase which contains the PROSITE consensus sequence between residues 240 and 261. Phylogenetic analysis (data not shown) of DitC indicates that it belongs to the I.3 family of dioxygenases, which includes enzymes with a preference for bicyclic substrates (14). This classification of DitC also conforms to the phylogenetic scheme proposed by Eltis and Bolin (11), since DitC has around 30% identity with several enzymes of this family (Table 2). However, DitC appears to form a new subfamily, since it has less than 54% identity to all extradiol dioxygenases found in GenBank.

Cell suspensions of the *ditC* knockout mutant strain BKME-93 transformed dehydroabietic acid and produced a yellow supernatant. The UV-visible absorbance spectrum of the supernatant showed maxima at λ 261 and 360 nm. GC-MS analysis of the supernatants of cell suspensions showed the transient accumulation of eight compounds. From the mass spectra of the methyl-ester derivatized metabolites, we assigned the following structures to three of the compounds (Fig. 1): compound IV, 7-hydroxydehydroabietic acid (M$^+$ 330, base peak 237); compound V, 7-oxodehydroabietic acid (M$^+$ 328, base peak 253); compound VIII, 7-oxo-11,12-dihydroxydehydroabietic acid (M$^+$ 374, base peak 299). The identity of compound V was confirmed by using a pure analytical standard, whereas compound IV was synthesized from the sodium borohydride reduction of compound V. A compound with the identical molecular ion as VIII was previously characterized as an intermediate of dehydroabietic acid degradation (6) and is presumably the oxidation product of the dihydrodiol intermediate from strain BKME-9, which was previously characterized (21).

Analysis of the other ORFs of the *dit* gene cluster. The two ORFs *ditD* and *ditH* encode putative proteins with weak sequence identity to HpcE and HpaG (28, 31) (Table 2). The putative stop codon of *ditC* is located 1 nucleotide upstream of the *ditD* start codon, and the predicted *ditE* start codon overlaps the putative stop codon of *ditD* by 4 nucleotides. Therefore, *ditCDE* are presumably cotranscribed. The coding sequences of *ditH* and *ditG* also overlap by 4 nucleotides, and these two genes are most likely cotranscribed. Three putative proteins encoded by *ditB*, *ditG*, and *ditI* showed similarity to proteins of the short-chain alcohol dehydrogenase/reductase (SDR) superfamily (15) (Table 2). The genes encoding the cleavage dioxygenase, DitC, and the ferredoxin, DitA3, are separated by *ditB* (Fig. 2). This location of *ditB* in the gene cluster suggests that it may encode a dihydrodiol dehydrogenase. However, its putative amino acid sequence shows little sequence similarity to known dihydrodiol dehydrogenases of aromatic degradation pathways (Table 2). A mutation in *ditB* did not impede dehydroabietic acid degradation (Table 3), and cell suspensions of the *ditB* knockout mutant strain did not accumulate pathway intermediates identifiable by GC-flame ionization detection (FID). In contrast, the *ditI* mutant accumulated one intermediate from 7-oxodehydroabietic acid. The mass spectrum (GC-MS) of this intermediate showed that it was not the dihydrodiol previously reported (6), but the structure of this intermediate was not elucidated. The analysis of the deduced amino acid sequences of *ditE* and ORF2 indicated similarity to membrane-bound permease proteins of the major facilitator superfamily (MFS) (Table 2). However, the two putative permeases showed no significant similarity to permeases of the aromatic acid:H$^+$ symporter family, which are frequently associated with aromatic acid catabolic pathways (26). Computer-assisted transmembrane topology predictions with TMpred and hydropathy plots of the deduced amino acid sequences identified 12 potential transmembrane helices in the ORF2 gene product and only 11 in DitE. From the predicted topology of the permease-like *ditE* gene product and its genetic locus, we postulate that it may be involved in the transport of diterpenoids into the cell, but there is no further evidence for this. The *ditF* gene encodes a 397-amino-acid protein with similarity to 3-ketoacyl coenzyme A (CoA) thiolases and sterol carrier proteins. SCP-X are multifunctional eukaryotic proteins with thiolase activity encoded in the N-terminal domain, which also promote the exchange in vitro of a variety of lipids

and sterols between membranes (35). The sterol carrier activity is encoded in the 143-amino-acid C-terminal domain of SCP-X (36), a domain that is not present in DitF.

DISCUSSION

We have previously shown that 7-oxodehydroabietic acid is produced from the incubation of dehydroabietic acid in dioxygenase-deficient strains of *P. abietaniphila* and that 7-oxodehydroabietic acid is the substrate for the diterpenoid dioxygenase DitA (21). The present study indicates that 7-oxodehydroabietic acid is also a key intermediate in the degradation of nonaromatic abietanes by strain BKME-9, since DitA$^-$ strains lack the ability to grow on abietic and palustric acids and accumulate 7-oxodehydroabietic acid when incubated with those substrates. There are some precedents for microbial degradation of cycloalkanes via aromatization and subsequent aromatic-ring cleavage. Both quinate (a substituted cycloalkane) and shikimate (a substituted cycloalkene) can be mineralized via the aromatic intermediate protocatechuate (43), and this pathway appears to be widespread among diverse microorganisms. Cyclohexanecarboxylic acid can be mineralized via the aromatic intermediates *p*-hydroxybenzoic acid and protocatechuic acid (7, 16), and this pathway exists in diverse microorganisms but is used by a minority of those growing on cyclohexane carboxylic acid (42). Despite these precedents, microbial degradation of cycloalkanes via aromatic intermediates appears to be unusual.

Like BKME-9, most of the resin acid-degrading bacteria previously isolated with dehydroabietic acid as the sole organic substrate also have the ability to grow on nonaromatic abietanes but not on pimeranes (25). This is consistent with a common abietane-specific degradation pathway for all of these organisms. Interestingly, 7-oxodehydroabietic acid has been detected in effluent biotreatment systems (46), suggesting that the abietane pathway reported here is ubiquitous. In light of the results presented in this study, the presence of this compound in biotreatment systems might be used as an indicator of biomass inhibition or sludge health with respect to its ability to degrade resin acids. If a common pathway is used for the microbial degradation of abietanes, this may have an important implication for the pulp and paper industry. Any adverse condition inhibiting this pathway in a wastewater biotreatment system would prevent the degradation of all abietane-type diterpenoids. This may result in the accumulation of resin acids at levels that are toxic and above regulatory criteria.

Pairwise comparison of the deduced amino acid sequences encoded by *dit* genes to similar proteins in databases showed at most 41% residue identity, with most proteins sharing 30% identity or less (Table 2). This meant that we could not deduce the function of most genes from their sequences, as is often the case for gene clusters encoding aromatic degradation pathways. Furthermore, a comparison of the genetic organization of the gene clusters encoding aromatic hydrocarbon oxidation pathways to that of the *dit* cluster revealed no similarity in the order or relative location of homologous genes, which suggests that these clusters are not closely related. In most instances, the three or four genes encoding the ring-hydroxylating dioxygenases for aromatic hydrocarbons are located in one transcriptional unit, eliminating the need for the coordination of gene expression (44). The genes encoding the diterpenoid oxygenase subunits, *ditA1* and *ditA2*, are located on a separate transcriptional unit from the genes encoding the electron transport components, *ditA3* and its putative ferredoxin reductase gene (Fig. 2). Similar unlinked organization was recently reported for genes encoding the components of dibenzo-*p*-dioxin (2) and of naphthalene/phenanthrene dioxygenases (17). Both studies demonstrated the substrate-dependent expression of the oxygenase components but did not show coordinated or constitutive expression of the electron transport proteins. In the case of the diterpenoid dioxygenase DitA, we have demonstrated the simultaneous induction of both the ferredoxin and oxygenase components. The multicomponent alkane hydroxylase of an *Acinetobacter* sp. is another example where the gene encoding the catalytic hydroxylase component (*alkM*) is distant on the chromosome from genes encoding its electron transport proteins (*rubA* and *rubB*) (13). In this case, expression of the catalytic hydroxylase component is regulated by alkanes (29), but expression of the electron transport proteins is constitutive (12).

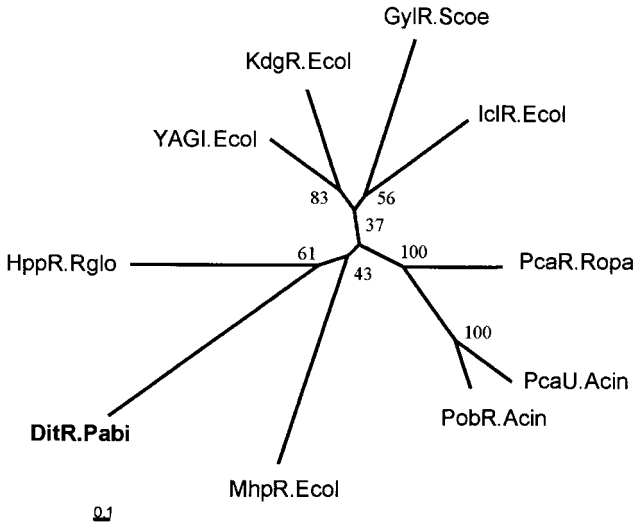

FIG. 6. Phylogenetic tree of IclR-type transcription regulators. The unrooted tree was generated with sequences aligned with ClustalX and by using the PHYLIP protein distance and neighbor-joining methods. The numbers on the branches represent bootstrap values of 100 replicate analyses. Accession numbers are as follows: for HppR.Rglo, U89712; for YAGI.Ecol, P77300; for KdgR.Ecol, P37728; for GylR.Scoe, P15360; for IclR.Ecol, AE000475; for PcaR-.Ropa, AF003947; for PcaU.Acin, L05770; for PobR.Acin, L05770; and for MphR.Ecol, P77569.

Phylogenetic analysis of the protein encoded by *ditR* indicates that it belongs to the IclR-like family of transcription regulators (Fig. 6). This family includes IclR, a repressor of the glyoxylate bypass operon in *E. coli* (41), GylR, an activator of the glycerol operon in *Streptomyces coelicolor* (38), and regulators of aromatic metabolism. The latter include PobR, an activator of the *p*-hydroxybenzoate hydroxylase enzyme PobA from an *Acinetobacter* sp. (9), and PcaR, an activator of protochatechuate degradation in *Pseudomonas putida* (30), as well as HppR and MhpR, regulators of 3-(3-hydroxyphenyl)propionic acid degradation in *Rhodococcus globerulus* and *E. coli*, respectively (3). Although we demonstrated that DitR positively regulates the expression of *ditA3*, the *P. abietaniphila* strain lacking a functional *ditR* maintained its ability to grow on abietanes (Table 3). This phenotype may be the result of the residual expression level of *ditA3* (Fig. 5). In addition, the *ditR* mutant reporter strain BKME-912 reproducibly responded, albeit at low expression levels, to the inducer 7-oxodehydroabietic acid (Fig. 5). These observations suggest that expression of *ditA3* may be controlled by at least one other mechanism. The examination of the nucleotide sequence upstream of *ditA3* identified a putative σ^{54}-promoter consensus sequence 89 bp upstream of the putative ATG start codon of *ditA3*.

Although we have no data confirming the involvement of the alternative sigma factor, σ^{54}, other than the consensus sequence, it is possible that the regulation of *ditA3* expression might also involve a σ^{54}-dependent regulator.

It was interesting to observe that structural analogues of dehydroabietic acid are gratuitous inducers of the dioxygenase genes. These analogues include isopimaric acid, a natural compound, and 12,14-dichlorodehydroabietic acid, a xenobiotic analogue resulting from pulp bleaching, neither of which is a substrate for the proposed pathway. This relaxed inducer specificity is common in the regulatory systems of catabolic pathways for the degradation of xenobiotic chemicals (8). However, diterpenes are natural compounds that have been present in nature for a long time. It might be hypothesized that since resin acid-degrading bacteria are unlikely to encounter an environment with only one species of diterpene, a lack of selective pressure to evolve a more specialized inducer response might have resulted in the response to a broad range of diterpenoid inducers.

Mutational analysis of several genes of the *dit* cluster failed to produce mutants that grow on dehydroabietic acid but do not grow on abietic and palustric acids (Table 3). Thus, the gene(s) encoding the enzyme(s) responsible for the aromatization of abietanes remains unidentified. The strain with a mutation in the gene encoding the extradiol cleavage dioxygenase produced a yellow supernatant from dehydroabietic acid in cell suspension assays. This yellow compound was not characterized, but we suspect that it was formed from the spontaneous oxidation of 7-oxo-11,12-dihydroxydehydroabietic acid to 7-oxodehydroabietic acid-11,12-quinone. The oxidation of the diol to a yellow compound would be similar to the previously described spontaneous oxidation of 1,2-dihydroxynaphthalene to 1,2-naphthaquinone (27).

The characterization of the abietane catabolic pathway of *P. abietaniphila* BKME-9 has significantly increased our understanding of the biodegradation of this industrially important and naturally abundant class of compounds. However, the present study also determined that the enzymes responsible for the early steps of the pathway were not located in the *dit* gene cluster. Therefore, work to identify the gene(s) encoding the enzymes for the conversion of abietic acid to the central metabolite, 7-oxodehydroabietic acid, is currently under way.

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